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A collagen hydrogel loaded with HDAC7-derived peptide promotes the regeneration of infarcted myocardium with functional improvement in a rodent model

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Running title: 7Ap-Collagen improved MI function

Abstract: Myocardial infarction (MI) leads to the loss of cardiomyocytes, left ventricle (LV) dilation, and cardiac dysfunction, eventually developing into heart failure. Most of the strategies for MI therapy require biomaterials that can support tissue regeneration. In this study, we hypothesized that the extracellular matrix (ECM)-derived collagen I hydrogel loaded with histone deacetylase 7 (HDAC7)-derived-phosphorylated 7-amino-acid peptide (7Ap) could restrain LV remodeling and improve cardiac function after MI. An MI model was established by ligation of the left anterior descending (LAD) coronary artery of C57/B6 mice. The 7Ap-loaded collagen I hydrogel was intramyocardially injected to the infarcted region of the LV wall of the heart. After local delivery, the 7Ap-collagen increased neo-microvessel formation, enhanced Sca-1^+ stem cell recruitment and differentiation, decreased cellular apoptosis, and promoted cardiomyocyte cycle progression. Furthermore, the 7Ap-collagen restricted the fibrosis of the LV wall, reduced the infarct wall thinning, and improved cardiac performance significantly at 2 weeks post-MI. These results highlight the promising implication of 7Ap-collagen as a novel candidate for MI therapy.

Keywords: myocardial infarction; collagen I hydrogel; 7Ap peptide; angiogenesis; anti-apoptosis; stem cell recruitment; cardiac functional recovery.

Abbreviations

left ventricle (LV), histone deacetylase (HDAC), myocardial infarction (MI), short open reading frame (sORF), 7-amino-acid peptide (7A), the phosphorylated 7A (7Ap), vascular progenitor cell (VPC), hypoxia/serum deprivation(H/SD), extracellular matrix (ECM), fetal bovine serum (FBS), echocardiographic (ECG), von Willebrand factor (vWF), left anterior descending coronary artery (LAD), hematoxylin andeosin (H&E), alpha-smooth muscle actin (α-SMA), stem cell antigen-1 (Sca-1).

1. Introduction

MI is an irreversible myocardium injury due to severe and accentuated reduction in coronary perfusion, making it one of the most serious ischemic heart diseases worldwide [1]. MI causes left ventricle (LV) remodeling, including progressive extracellular matrix (ECM) degradation, LV wall thickness increase, scar formation, and finally transition to heart failure in the late phase [2]. Currently, surgical intervention, such as bypass and stenting operation, is the first choice for MI patients. Because the surgery can only attenuate the symptoms, the regeneration of infarct tissue is less effective [3].

Various bio-activate molecules, such as growth factors, functional peptides, and stem cells have been widely applied for ischemic heart regeneration [4-6]. Histone deacetylases (HDACs) are a family of enzymes that remove acetyl groups from N-acetylated lysine residues on histones and HDACs play an important role in promoting chromatin compaction and modulating gene transcription, hence regulating many aspects of cardiovascular diseases, including MI from pathogenesis to therapeutics [7-9]. HDAC7 belongs to the class II HDAC family, and is specifically expressed in the vascular endothelium and contributes to the maintenance of vascular integrity during early embryogenesis [10, 11]. There are several splicing variants in *HDAC7* mRNAs. One of the mouse *Hdac7* transcript variants has a short open reading frame (sORF) located in the 5' terminal non coding area, encoding a 7-amino-acid peptide (7A, MHSPGAD). The 7A could transfer the phosphate group from the phosphorylated Ser393 site of MEKK1 to the site of 14-3-3 γ protein, forming an MEKK1-7A-14-3-3 γ signaling pathway. The phosphorylated 7A (7Ap, MH[pSer]PGAD) was shown to increase stem cell migration, proliferation, and differentiation, especially that of vascular progenitor cells (VPCs). 7Ap enhanced angiogenesis, ameliorated vascular injury and promoted blood perfusion recovery in in a femoral artery injury model and a hindlimb ischaemia model [12]. According to the properties of 7Ap, in this study, we speculated that the peptide should greatly contribute to MI therapy.

Despite the ongoing research activities conferred on peptides, several central concerns should be addressed. For instance, peptides exhibit short half-life and can be easily degraded by endogenous peptidases, whereas tissue engineering has provided a choice to overcome these shortcomings. Currently, various bio-active elements combined with biomaterials have been applied for ischemic heart regeneration, and these biomaterials are often fabricated into injectable forms [4, 13]. Hydrogel, one kind of injectable biomaterial, has the advantages of minimizing invasion, maintaining cardiac structure, and acting as vehicles to deliver bio-active elements *in situ* [14, 15]. Numerous natural biomaterials have been used for cardiac tissue engineering such as collagen, gelatin, laminin, chitosan and hyaluronic acid [16]. Collagen I is one kind of ECM proteins and it can assemble into a 3D polymeric network with a high-water content. Collagen I based hydrogel has been used extensively as scaffolds for tissue engineering or as delivery systems for stem cells and/or therapeutic agents, because it can provide a favorable microenvironment for cell survival and proliferation. Moreover, it constitutes a functional platform to prolong the retention of the bio-active elements [17, 18]. Owing to its high permeability and biocompatibility, Injectable collagen I hydrogel has already been applied for MI therapy in animal models [19, 20].

The aim of this study was to develop a 7Ap-loaded collagen I hydrogel that provides a conducive environment for cardiac tissue repair and regeneration. Locally released 7Ap from the collagen hydrogel serves as a bioactive factor with anti-apoptotic and angiogenic function; it could further promote the mobilization and recruitment of endogenous Sca-1^{+} stem cell to the site of injury. Collectively, intra-myocardial delivery of 7Ap-loaded collagen will enhance the revascularization around the site of injury and contribute to MI therapy.

2. Materials and Methods

2.1 Preparation of peptides and hydrogels

The 7Ap peptide was synthesized by Genscript (Piscataway, USA). The preparation of collagen I hydrogel was reported previously [14]. Briefly, highly hydrated collagen was produced by adding 1.1 ml of saline to 0.9 ml of sterile rat tail collagen type I (BD Biosciences, USA) in acetic acid (3.84 mg/ml, Millipore, US). The resulting 2-ml collagen/saline mixture was mixed well, and the final pH was adjusted to 7.4 using 0.1 M of NaOH. The 7Ap-collagen was prepared by mixing peptide stock solution with collagen at 4°C. The final concentrations for 7Ap and collagen I were 0.1 μg/kg and 3.3 mg/ml, respectively. For animal experiments, 20 μl of the 7Ap- collagen was injected into the myocardium of the infarcted heart. The collagen-based hydrogel was liquid at the time of intra-myocardial injection but shows gelation at physiological temperature (37°C) within 10 minutes.

2.2 Characterization of 7Ap-loaded collagen hydrogel

(a) The morphology of 7Ap-loaded collagen hydrogel

After lyophilization, the 7Ap-collagen was examined using scanning electron microscopy (Quanta 200; FEI, Brno). Briefly, the freeze dried hydrogel was first cut to display their inner structures. After spraying a gold layer, the cross sections were visually observed by SEM.

(b) *In vitro* release of 7Ap

Hydrogel with 200mg of fluorescein isothiocyanate (FITC)-conjugated 7Ap (7Ap-FITC) (n=3) was incubated in 1 ml of phosphate-buffered saline (PBS) at 37°C. At each time point, supernatants were collected and stored at 4°C for analysis, followed by the addition of 1 ml of fresh PBS. The amount of 7Ap-FITC in the supernatant was measured by fluorescence intensity at 490 nm using a PerkinElmer EnSpire (PerkinElmer LLC). A standard curve was carried out using a series of known 7Ap-FITC contents in PBS solution. All experiments were replicated 3 times.

2.3 H9C2 cell culture under hypoxia/serum deprivation (H/SD) conditions

The rat heart tissue-derived H9C2 cardiac myoblast cell line was purchased from American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 100 U penicillin/streptomycin at 37°C in a humidified 5% CO_2–95% O_2 incubator. To induce ischemia, cells cultured in a simulated ischemia solution (glucose-free Tyrode solution containing 10 mM2-deoxy-D-glucose and 10 mM of sodium dithionite) were exposed to hypoxia (<1% O_2) by placing the plates in a hypoxia chamber for 2 h.

To test the effect of 7Ap on cardiac ischemia injury *in vitro*, H9C2 were seeded in 6-well plates at a density of 5×10^4 cells per well 24 h prior to the experiment. The cells were assigned to 3 groups randomly as follows: normal group (cells were cultured in DMEM+10% FBS, in a 95% O_2 atmosphere) n=5; H/SD group (cells were cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5; and 7Ap + H/SD group (cells were exposed to 1 ng/ml 7Ap and cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5. After 2 hours, cells in each group were harvested and prepared for fluorescence-activated cell sorting (FACS) and western blot experiments.

2.4 FACS examination

Cells were harvested after the incubation period and washed in cold PBS. The cells were stained with Annexin V (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, BD, US) according to the protocol provided. Finally, the numbers of Annexin V-positive cells were examined by FACS (Calibur, USA). Normal group (positive control) (cells were cultured in DMEM+10% FBS, in a 95% O_2 atmosphere) n=5; H/SD group (negative control) (cells were cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5; and 7Ap + H/SD group (cells were exposed to 1 ng/mL 7Ap and cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5. All experiments were replicated 3 times.

2.5 Western blot analysis

Whole cell lysates were prepared with protein concentrations measured by Bio-Rad reagents (Hercules, USA). Fifty micrograms of cell lysates were applied on (10-12%) sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were probed with primary antibodies overnight at 4°C. Each primary antibody binding was detected with a horseradish peroxidase (HRP)-conjugated secondary antibody and visualized by the enhanced chemiluminescence (ECL) method. Band intensity was normalized to the signal intensity of internal controls using Image-Pro Plus software. Normal group (positive control) (cells were cultured in DMEM+10% FBS, in a 95% O_2 atmosphere) n=5; H/SD group (negative control) (cells were cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5; and 7Ap + H/SD group (cells were exposed to 1 ng/mL 7Ap and cultured in simulated ischemia solution, in a 1% O_2 atmosphere), n=5. All experiments were replicated 3 times.

2.6 MI model and experimental groups

For this study, C57/B6 (female, 20 ± 2 g), were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). All animal experiments were carried out using the guidelines established by the Tianjin Committee of Use and Care of Laboratory Animals and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. During the surgery, the body temperature of the mouse was maintained at 37.5°C ± 0.5 using a heating table. During the first 4 days, mice were offered NovaMin sulfone (metamizol) (10 drops/300 mL water) for pain-free, and the drinking water was replaced daily. The animals' conditions, such as activity, behavior, lethargy, lack of appetite, and hair texture were assessed each day. The mice were euthanized when they exhibited significant impairments of their conditions.

Mice were randomly assigned to 4 groups as follows: No MI-operation, n = 5; MI-saline, n = 6; MI-collagen, n = 10; MI-7Ap-collagen, n= 12.

The MI model was induced by permanent ligation of the left anterior descending coronary artery (LAD) as previously described [5]. Briefly, mice were anesthetized through inhalation of isoflurane (1–1.5%) in O_2, followed by incubation and mechanical ventilation using an anesthetic machine (Hallowell EMC MicroVent 1, Pittsfield, MA, USA). After the mice entered a relaxed state without breath depression and dysphoria, the LAD was permanently ligated using a 7–0 silk suture via a left thoracotomy at the fourth intercostal space. Infarction was considered successful following the visual appearance of pale discoloration. Two days after injection, mice were assessed by Echocardiographic (ECG) and only those exhibiting 38%-45% LV-EF were included in the study (Supplementary Fig. S1).

After ligation, 20 μl of 7Ap-collagen, collagen, or saline were administered in two injections into two areas adjacent to the infarcted zone with a 30-gauge needle (BD Biosciences, USA). Afterward, the chest wall and skin were closed. When the anesthesia was stopped, the animals woke up immediately (normally in approximately 1–2 min). No MI operations were performed on another 5 mice without LAD occlusion.

2.7 ECG assessment

Mice were anesthetized through inhalation of isoflurane (1–1.5%) in O_2 and ECG was performed on mice using a VisualSonics echocardiographic system equipped with a 30-MHz transducer (RMV-707B; Toronto, Canada) 2 days and 2 weeks post-MI. The motion of the myocardium overtime was recorded using B and M Mode at the level of the papillary muscle. The continuous pressure and volume signals were monitored and analyzed using the Vevo2100 V3.0.0 Software (VisualSonics Inc., Canada). All analyses were performed by a single investigator who was blinded to the treatment groups. Heart function indices including LV Stroke Volume, LV Ejection Fraction, and

LV Cardiac Output were calculated based on the ECG records. All analyses were performed by a single investigator who was blinded to the treatment groups.

2.8 Histologic analysis

After ECG measurement, mice were euthanized by 5% potassium chloride (KCL) perfusion. Overdose of 5% KCL may cause cardiac conduction blockages in the diastolic phase. Then, the hearts were isolated, embedded in liquid nitrogen, and cut into 5-μm-thick sections. Five consecutive sections at 1-mm intervals were stained with Masson's Trichrome for infarct size, infarct wall thickness, and collagen deposition evaluation as reports previously [21, 22]. The infarct size (2 sections/mouse) was calculated from sections stained with Masson's Trichrome. Briefly, the lengths of the infarcted surfaces, involving both epicardial and endocardial regions, were measured. And the infarct size was determined as a percentage of the total LV circumference. Infarct wall thickness (2 Sections/mouse) was derived from an average of 3 measurements taken throughout the respective regions. To evaluate fibrosis (2 Sections/mouse), the methyl blue positive regions of collagen deposition were examined in 10 randomly chosen fields per section using Image-Pro Plus software. Collagen density was expressed as the ratio of collagen deposition to myocardial tissue in percentage. All data were analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Silver Spring, MD, USA).

2.9 Immunofluorescent and immunohistochemistry evaluation

Endothelial cells contain von Willebrand factor (vWF) in vesicles in the quiescent state, enabling the labeling of endothelial cells. Arterioles usually have one or two layers of smooth muscle as muscular walls, thus the alpha-smooth muscle actin (a-SMA) can be used for arteriole staining. Isolectin IB4 is a glycoprotein and has particular strong affinity for capillaries. In this study, to determine the angiogenic effects of 7Ap, frozen sections were immunostained with primary antibodies against vWF (Dako,

Denmark; 1:200 dilution) for vessels (diameter > 50 μm), a-SMA (Boster Biotechnology, China; 1:200 dilution) for arterioles (diameter < 50 μm), and isolectin-IB4 conjugated with Alexa Fluor 488 (Molecular Probes, USA; 1:200 dilution) for capillaries. After primary antibody incubation at 4°C overnight, the secondary antibodies of goat anti-rabbit Alexa-Fluor 488 or goat anti-rat Alexa-Fluor 488 (Invitrogen, USA; 2mg/mL, 1:1000 dilution) were incubated with sections for 2 hours. To determine stem cell recruitment, frozen sections were immunostained with primary antibody against stem cell antigen-1 (Sca-1; Abcam, USA; 1:200 dilution). After incubation of primary antibodies overnight at 4°C, the secondary antibodies of rabbit anti-mouse Alexa-Fluor 488 (Invitrogen, USA; 2mg/mL, 1:1000 dilution) was incubated with sections for 2 h. Meanwhile, to determine the cardiomyocyte proliferation, frozen sections were co-immunostained with primary antibody against Ki67 (Abcam, USA; 1:200 dilution) and Troponin T (Abcam, USA; 1:200 dilution). The secondary antibodies of rabbit anti-mouse Alexa-Fluor 546 (Invitrogen, USA; 2mg/mL, 1:1000 dilution) and goat anti-rabbit Alexa-Fluor 546 (Invitrogen, USA; 2mg/mL, 1:1000 dilution) were incubated with sections for 2 hours. The sections without incubation with primary antibodies were used as negative controls. The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) containing mounting solution (DAPI Fluoromount G, Southern Biotech, England). The samples of the vessels, arterioles, and capillaries, Sca-1^+ cells were observed using a fluorescence microscope (Zeiss Axio Imager Z1, Germany). The samples of $Ki67^+$/troponin T^+ cells and Troponin T^+ cardiomyocytes were analyzed using LSM 780 confocal microscopy (Carl Zeiss). The infarct areas, border zones, and remote areas were defined as reported previously [13, 22, 23]. The numbers of vessels, arterioles, and capillaries, Sca-1^+ cells, $Ki67^+$/troponin T^+ cells, and Troponin T^+ cardiomyocytes were counted in 10 randomly chosen high-power fields (HPF) of the border zone. Results were expressed as cells per mm^2. All morphometric studies were performed by 2 examiners who were blinded to the treatment.

TUNEL staining of frozen embedded tissue of all the experimental groups was performed by using a TUNEL apoptosis assay kit (Beyotime Biotechnology, China). For each slide, 5 separated fields were randomly selected, and cells with clear nuclear labeling were defined as TUNEL-positive cells. The apoptotic index was calculated as the number of TUNEL-positive cells at the border zone of infarct area. Images were observed under an inverted microscope (Nikon Eclipse TE2000-U Kanagawa, Japan).

2.10 Statistical analysis

All data were presented as means±SEM. An independent t-test was used for two-group comparisons, and one-way analysis of variance (ANOVA) was used for multiple-group comparisons, Least significant difference (equal variances) and Dunnett's T3 (non-equal variances) post hoc tests were used for testing the differences between groups. A p-value of $p< 0.05$ was considered to be statistically significant.

3. Results

3.1 7Ap increased H9C2 cell survival under hypoxia and serum deprivation (H/SD)

MI induces hypoxia and blood supply in the infarcted area, causing cardiomyocyte apoptosis. To test whether 7Ap could increase cardiomyocyte survival, we first assessed its effect on H/SD-induced apoptosis *in vitro* with the rat cardiac myoblast cell line H9C2. As shown in Fig.s 1A and 1B, H/SD-induced apoptosis in H9C2 cells, which was significantly reduced by the presence of 7Ap, as revealed by FACS analysis. Further experiments with western blot analysis demonstrated that 7Ap treatment increased Bcl2 and Akt phosphorylation but decreased cytochrome C (Fig. 1C), which may contribute to the 7Ap-mediated cell survival protection.

3.2 Characterization of 7Ap-7Ap-loaded collagen hydrogel

Hydrogel containing bio-therapeutic materials, such as peptides, have been widely used in MI therapy [24]. In this study, 7Ap was physically incorporated into the hydrogel (Fig.s 2A-D). Because of the thermo-sensitive properties of the collagen, the

higher temperature resulted in its faster gelation (Fig.s 2A and 2B). It took 7.51 min to form hydrogel at 37°C. Moreover, the morphologic structure of 7Ap-collagen was examined by scanning electron microscopy (Fig. 2C). Additionally, the representative release curve of 7Ap from hydrogel was shown in Fig. 2D. The release rate was rapid during the initial 32 h, and cumulative release was 32.0% of the theoretical value, then slowed down to a steady rate. The total release of 7Ap reached about 43.5% after 128 hours and no burst release was observed during the detection period.

The bioactivity of 7Ap released from collagen hydrogel also evaluated. It still has the function of anti-apoptosis after collagen encapsulation (Supplementary Fig. S2).

3.3 Local delivery of 7Ap via 7Ap-collagen improved LV function following MI

To test whether 7Ap had therapeutic protection in vivo, MI was introduced in mice via LAD ligation. Two injections of 7Ap-collagen were performed on both sides of ligated area (Fig. 2E). No MI operation, saline, and collagen were included as controls.

Meanwhile, the *in vivo* localization and retention of 7Ap at different time-points were analyzed by using FITC-labeled peptides. The peptides remained in the ischemic zone for up to 5 days and gradually removed from the heart with the degradation of collagen hydrogel (Supplementary Fig. S3).

Fourteen days post-surgery, LV function was evaluated using ECG. B and M-mode echocardiography showed important indicators for blood pumping function, such as ejection fraction and cardiac output. In comparison to the no MI group, the infarcted mice displayed a decrease in blood pumping function, which was significantly improved by the local delivery of 7Ap. As shown in Fig. 3A, 7Ap treatment restored LV function significantly. As expected, infarction increased the ratio of heart weight to body weight (Fig. 3B) (Values: no-MI, 4.8± 0.1, saline, 5.1 ± 0.2; collagen, 4.9 ± 0.2; 7Ap-collagen, 4.9 ± 0.1). As reported previously [6], infarction significantly decreased LV stroke volume (Fig. 3C) (Values: no-MI, 45 ± 8, saline, 23 ± 3; collagen, 25 ± 5; 7Ap-collagen, 38 ± 6), LV fractional shortening (Fig. 3D)

(Values: no-MI, 34 ± 3, saline, 21 ± 4; collagen, 21 ± 2; 7Ap-collagen, 30 ± 5), LV ejection fraction (Fig. 3E) (Values: no-MI, 68 ± 6, saline, 37 ± 5; collagen, 42 ± 4; 7Ap-collagen, 57 ± 4), and LV cardiac output (Fig. 3F) (Values: no-MI, 22 ± 3, saline, 10 ± 2; collagen, 11 ± 1; 7Ap-collagen, 16 ± 1), which were significantly improved by the delivery of 7Ap, indicating a recovery in blood pumping and improvement in ventricular filling. We also evaluated the LV end-diastolic diameter (LVEDd) and LV end-systolic diameter (LVEDs) of hearts post-MI (Fig.s 3G and H). And 7Ap delivery reduced LVEDd significantly (Fig. 3G) (Values: no-MI, 3.8 ± 0.1, saline, 4.4 ± 0.4; collagen, 4.0 ± 0.2; 7Ap-collagen, 3.9 ± 0.2), whereas the LVEDs was not markedly affected by 7Ap treatment (Fig. 3H) (Values: no-MI, 2.6 ± 0.3, saline, 3.8 ± 0.5; collagen, 3.5 ± 0.5; 7Ap-collagen, 3.3 ± 0.6). Thus, 7Ap treatment significantly enhanced contractility, decreased stiffness, and increased elasticity of the LV.

3.4 Local delivery of 7Ap increased angiogenesis in the infarcted region

Angiogenesis plays an essential role in heart function recovery following MI. Therefore, we assessed new vessel formation to explore whether 7Ap-mediated protection occurred through angiogenesis enhancement. Capillaries in the MI border region were examined by Isolectin IB4 staining. The capillary density in the 7Ap-treated hearts was significantly higher than that in the saline and collagen groups at 14 days (Fig. 4A) (Values: no-MI, 14630 ± 755, saline, 10700 ± 330; collagen, 10501 ± 541; 7Ap-collagen, 12310 ± 752). Indeed, small arterioles (diameter < 50 μm) reduced significantly in the ischemic myocardium. Conversely, cardiac arteriole density was preserved moderately in the border zone of the LV in the 7Ap-collagen group (Fig. 4B) (Values: no-MI, 47 ± 9, saline, 23 ± 8; collagen, 26 ± 12; 7Ap-collagen, 40 ± 8). Microvascular density in the ischemic myocardium was assessed by vWF immunostaining 14 days following 7Ap delivery (Fig. 4C) (Values: no-MI, 6 ± 8, saline, 29 ± 11; collagen, 46 ± 11; 7Ap-collagen, 118 ± 23). In comparison to other MI-treated groups, 7Ap increased the vessel density approximately

3-fold in the treated hearts. These results suggest that 7Ap treatment could improve the angiogenic properties in the post-ischemic heart (Fig.s 4D-F).

LAD ligation results in MI and causes post-infarction cardiac remodeling, which exhibits typical histological changes, including progressive ventricular chamber dilation, thinning of the LV wall, and extensive collagen deposition (Fig. 5). We calculated the infarct size and LV anterior wall thickness based on 2 Masson's trichrome-stained sections. Although the infarct size did not differ significantly among different MI-treated groups, LV infarct wall thickness was significantly increased in the 7Ap treated group compared to other MI-treated groups (Fig. 5A). Computerized planimetry of heart cross-sections indicated that more collagen deposition occurred in all MI-treated groups (Fig. 5B), whereas the 7Ap-collagen group showed markedly decreased collagen deposition compared to other MI-treated groups (Fig.s 5C-E). These data suggest that 7Ap delivery maintains the LV geometry well and reduces the adverse ventricular remodeling.

3.5 Local delivery of 7Ap inhibited apoptosis, enhanced Sca-1$^+$ stem cell recruitment and differentiation, and stimulated cardiomyocyte cycle progression

Apoptotic effects were evaluated in peptide-treated animals at 14 days post-MI. More apoptotic cells (TUNEL positive cells) were observed in MI-treated groups 14 days post MI, whereas there were only a few TUNEL-positive cells in the border zone of the 7Ap-treated hearts, and there was significant difference in TUNEL-positive cells between 7Ap and other MI-treated groups (Fig. 6A). These findings suggest that the anti-apoptotic effects of 7Ap delivery may be one important mechanism responsible for the beneficial cardiac effects.

Sca-1$^+$ stem cell homing and differentiation after 7Ap injection was evaluated by immunohistochemical staining (Fig. 6B). The Sca-1$^+$ stem cell numbers were significantly increased in the 7Ap-collagen group compared to the saline and collagen groups. Further experiment with double immunofluorescence staining using CD31 (red) and Sca1 (green) antibodies (Supplementary Fig. S4) revealed that part of the CD31

cells were also Sca1$^+$, indicating Sca-1$^+$ stem cells can differentiate and contribute to vascular remodeling following cardiovascular injury. Meanwhile, the delivery of 7Ap dramatically increased Ki67$^+$/troponin T$^+$ cell numbers and the ratio of Ki67$^+$/troponin T$^+$ cell vs Troponin T$^+$ cardiomyocytes was increased significantly at the border zone of MI (Fig.s 7A-C, Supplementary Fig. S5 and Fig. S6). These phenomena reflect that 7Ap may not only recruit Sca-1$^+$ stem cells into the infarcted area, but also stimulate the cardiomyocyte cycle progression, increasing the pool of proliferating cardiomyocytes.

4. Discussion

The major goal of cardiac tissue engineering is to create a favorable microenvironment in ischemic regions in order to regenerate the damaged heart tissue and preserve the heart function. Stem cells, growth factors, and other bio-activate elements have been applied to treat ischemic hearts [25-27]. Numerous functional/therapeutic peptides have been widely applied for cardiac disease research due to their advantages of easy synthesis, low toxicity, and rapid tissue absorption [24, 28].

A therapeutic bio-activate peptide, 7Ap, which is derived from the sORF of *Hdac7* and functions as a phosphate transfer carrier in cellular signal transduction plays a vital role in promoting endothelial progenitor cells, especially Sca-1$^+$ stem cell migration and self-renewal capacity. Meanwhile, 7Ap may induce capillary vessel network formation and promote blood perfusion recovery. In this study, we evaluated its potential in tissue repair after MI by incorporating 7Ap peptides into the rat tail-derived collagen.

We have demonstrated that the 7Ap-collagen can promote the micro-vessel formation in ischemic areas, thus supporting cellular survival, limiting the degree of cardiac fibrosis, preventing the LV negative remodeling, and finally improving heart function in the murine MI model.

In the 7Ap-collagen group, the survival rate of cells was significantly higher

compared to collagen and saline groups. Consistently, the *in vitro* data revealed the peptide's anti-apoptotic function in H9C2 cells. Our results proved that the anti-apoptotic effects of 7Ap are partly due to its inactivating cytochrome 3 protein, activating Bcl-2 protein, and stimulating ERK phosphorylation, which is an important component of the MEK-ERK pathway.

Recent studies have supported the idea that cardiomyocytes in adult mammals can be stimulated to re-enter the cell cycle [29, 30]. Meanwhile, our *in vivo* results demonstrated that 7Ap dramatically increased Ki67 protein expression at the border zone of MI, which regulates cardiomyocyte transition from the G2 (secondary growth) phase to the S phase during cell cycle progress. Cardiomyocyte in S phase enhances DNA replication and suggests cells are "primed" for proliferation. At the infarct border zone, Ki67 expression may have the potential to increase the pool of proliferating cardiomyocytes.

A higher cellular survival rate and cardiomyocyte cycle progression synergism increased LV wall thickness. The benefits of increased LV wall thickness are the altered properties of surrounding tissue and decreased LV wall stress. Less LV wall stress results in reduced end diastolic volume. The decrease in LV wall stress is also thought to protect the vulnerable myocardium, reducing apoptosis and infarct expansion, and thus preventing pathologic LV remodeling.

In addition, the current study has shown that intra-cardiac 7Ap injection may recruit the Sca-1^+ stem cell migration to the ischemic myocardium. Sca-1^+cell population has the potential of promoting the neo-vessel formation, modulating the cardiac progenitor cell proliferation and further restoring the cardiac function [31, 32]. Furthermore, implantable collagen may supply a 3D microenvironment for homing endogenous Sca-1^+ stem cell retention, survival, and differentiation.

Our research has shown that 7Ap treatment improved the capillary density and promoted neovascularization at the border zone of the ischemic heart. In a parallel study [12], we have demonstrated that the inclusion of 7A, especially the phosphorylated version, 7Ap, significantly increased endothelialization. The majority of the endothelial

cells derived from the local resident Sca-1$^+$ stem cells of the surrounding tissues, because 7Ap can serve as a chemoattractant for Sca-1$^+$ stem cells and direct their differentiation toward the EC lineage. In this study, we proved that the benefit of vessel formation around the MI area is associated with the resident Sca-1$^+$ stem cell migration and differentiation (Fig. S6). Additionally, increased angiogenesis post-MI indicated that 7Ap treatment could be more advantageous in long-term MI therapy if it can sustain the degree of mature vasculature formation.

Myocardial fibrosis is a key contributor to cardiac dysfunction after MI. The pathological accumulation of fibrosis at the infarcted site might present a physical barrier that impairs the migration and repair of stem cells [33]. Fibrosis analysis showed that 7Ap delivery significantly decreased the fibrosis size at the border of the infarcted zone as compared to other MI-treated groups. The 7Ap has a positive effect on vascularization, and reduced extent of fibrosis could be the downstream of this beneficial effect, as well as the reconstruction of the LV.

There are also several limitations in this study. First of all, we only implanted 7Ap collagen hydrogel for up to 2 weeks. Long-term evaluation will elucidate its potential for the regeneration of infarcted myocardium. In addition, the exact mechanism of 7Ap leading to cardiac regeneration in MI has not been investigated in this study. Finally, the clinical relevance associated with a mouse MI model is absent. In the clinical setting, factors such as the LV volume and structure, biomaterial injection volume, and method for injection will be very different from those in the small animal model.

5. Conclusions

In summary, we developed and characterized a bioactive 7Ap-loaded collagen hydrogel for *in situ* tissue repair in the settings of MI. *In vivo*, intra-myocardial delivery of 7Ap-loaded collagen in a mouse MI model decreased cellular apoptosis, enhanced Sca-1$^+$ stem cell recruitment and differentiation, and increased neovascularization formation. As a result, increased infarct wall thickness and improved heart function, and finally attenuated adverse cardiac remodeling have been observed for up to 2 weeks. This study demonstrates the potential of the implementation of 7Ap for ischemic

cardiac tissue repair. Additional studies are needed in order to understand the mechanisms underlying the myocardial regeneration stimulated by the 7Ap-loaded hydrogel and to further improve their therapeutic efficacy.

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Conflict of interest

Authors declare no conflict of interest.

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Figure Legends

Fig. 1. 7Ap treatment suppressed H/SD-induced apoptosis in H9C2 cells. H9C2 cardiac myoblasts were treated with or without 1 ng/ml of 7Ap under H/SD conditions for 2 h, followed by FACS analysis (A: FACS images and B: quantitative analysis of Annexin V-positive cells in each group) and western blot analysis (C, left panel shows the western blot images, right panel shows the relative ratio of the target protein to GAPDH with that of normal group set as 1.0). Data presented were representative images or mean SEM of 3 independent experiments. *: $p< 0.05$.

Fig. 2. Preparation and MI injection of 7Ap-loaded collagen hydrogel. (A) Quantification of the gelation time of the collagen I hydrogel at different temperatures (n = 3 per temperature). (B) The morphology of collagen before and after gelation. (C) Scanning electron microscope image of collagen hydrogel. (D) The cumulative release

of 7Ap from the collagen hydrogel. (E) MI surgery and the intra-myocardial delivery of 7Ap-loaded collagen following MI. After ligation (red cross), 7Ap-collagen was administered in two injections (full black arrows) into two areas adjacent to the infarcted zone (gray circle), left panel.

Fig. 3. Intramyocardial delivery of 7Ap-collagen restored cardiac functions 14 days post-MI. (A) Representative echocardiographic 2D images of hearts of each group. (B) heart weight/body weight, (C) LV stroke volume, (D) LV fractional shortening, (E) LV ejection fraction, (F) cardiac output, (G) LV dimensions at end-diastole, (H) LV dimensions at end-systole. *$p < 0.05$. No MI (n=5), saline (n=6), collagen (n=6), 7Ap-collagen (n=7).

Fig. 4. Intramyocardial delivery of 7Ap-collagen enhanced neo-vascularization 14 days post-MI. (A) Representative images of isolectin IB 4 (green)-stained capillaries at the border zone, (400×). (B) Representative images of αSMA (green)-stained arterioles at the border zone (200×). (C) Representative images of vWF (green)-stained blood vessels at the border zone (400×). (D) Qualification of capillary, arteriole, and blood vessel density at the border zone of ischemic myocardium. Data shown as mean SEM; *$p < 0.05$. No MI (n=5), saline (n=6), collagen (n=6), 7Ap-collagen (n=7).

Fig. 5. Intramyocardial delivery of 7Ap-collagen alleviated MI progression and collagen deposition 14 days post-MI. (A) Representative heart cross-sections stained with Masson's trichrome 14 days post-MI. (B). Representative heart cross-sections stained with Masson's trichrome at the border zone 14 days post-MI. (C) Quantitative analysis of infarct size and anterior wall thickness 14 days post-MI. (D) Quantitative analysis of collagen deposition at the border area 14 days post-MI. Data shown as mean SEM; *$p < 0.05$. No MI (n=5), saline (n=6), collagen (n=6), 7Ap-collagen (n=7).

Fig. 6. 7Ap-collagen injection reduced apoptosis and improved Sca-1$^+$ stem cell recruitment 14 days post-MI. (A) Representative images of TUNEL-positive nuclei (brown dots; white arrows) at the border zone (200×). (B) Representative images of

Sca-1$^+$ (green) stem cells at the border zone (400×). (C-D) Quantitative analysis of TUNEL-positive nuclei and Sca-1$^+$ stem cells at the border area 14 days post-MI. Data shown as mean SEM; *p < 0.05. No MI (n=5), saline (n=6), collagen (n=6), 7Ap-collagen (n=7).

Fig. 7. 7Ap-collagen injection activated Ki67$^+$/troponin T$^+$ cell cycle progression 14 days post-MI. (A) Representative images of Ki67$^+$/troponin T$^+$ cardiomyocytes at the border zone (400×). White arrows indicate the Ki67$^+$ nuclei. (B) Quantitative analysis of Ki67$^+$/troponin T$^+$ cell at border area 14 days post-MI. (C) Quantitative analysis of the ratio of Ki67$^+$/troponin T$^+$ cell vs Troponin T$^+$ cardiomyocytes at border area 14 days post-MI. Data shown as mean SEM; *p < 0.05. No MI (n=5), saline (n=6), collagen (n=6), 7Ap-collagen (n=7).